Effects of KB-2796, a New Calcium Antagonist, and Other Diphenylpiperazines on [3H]Nitrendipine Binding

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Abstract—The effect of KB-2796, a new diphenylpiperazine calcium antagonist, on [3H]nitrendipine ([3H]NTD) binding was investigated in synaptosomal membranes prepared from the guinea pig cerebral cortex. KB-2796 inhibited [3H]NTD binding in a dose-dependent manner with an IC50 value of 86 nM. In this respect, KB-2796 was the most potent among the diphenylpiperazine derivatives tested. Saturation binding data indicated that this inhibition resulted from a decrease in the binding affinity without changes in the maximal number of binding sites. KB-2796, however, significantly increased the dissociation rate constant of [3H]NTD from radiolabeled membranes. This finding suggests that KB-2796 inhibits [3H]NTD binding by a negative heterotropic allosteric mechanism. Other diphenylpiperazines tested also showed similar inhibitory properties. Diphenylpiperazines may act at a site, which is different from the 1,4-dihydropyridine binding site, on the voltage-dependent calcium channel.

Calcium antagonists are structurally heterogeneous organic compounds that are generally classified into four major groups: 1) 1,4-dihydropyridines (1,4-DHPs): nifedipine, nicardipine and nimodipine, 2) phenylalkylamines: verapamil, 3) benzothiazepines: diltiazem and 4) diphenylpiperazines: flunarizine and cinnarizine (1). The structural heterogeneity of these compounds is consistent with their different clinical, pharmacological and electrophysiological effects (2-4). KB-2796, 1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride, is a newly synthesized calcium antagonist which has a diphenylpiperazine moiety (5). KB-2796 has been reported to selectively inhibit the contraction of cerebral arteries induced by K+ and prostaglandin F2α (6) and to markedly increase cerebral blood flow in immobilized cats (7).

Recently, high affinity binding sites for [3H]nitrendipine ([3H]NTD), which may be associated with voltage-dependent calcium channels, have been demonstrated in membrane preparations obtained from the brain, heart, smooth muscle and skeletal muscle (8, 9). The structurally diverse calcium antagonists, i.e., (+)-verapamil, d-cis-diltiazem, (+)-bepridil, and their related compounds, are known to allosterically modulate the binding of [3H]NTD through their own distinct binding sites which are possibly located on the same voltage-dependent calcium channels (10-14).

The purpose of this study was to characterize the interaction of KB-2796 and other diphenylpiperazine derivatives with the 1,4-DHP binding site in cerebral cortex membranes. In addition, we examined the effects of diphenylpiperazines on vertebral blood flow in anesthetized dogs.

Materials and Methods

Membrane preparations: Male Hartley guinea pigs (350–500 g) were killed by decapitation, and the cerebral cortex was rapidly removed. The cerebral cortex was homogenized in 10 volumes of 0.32 M sucrose using a Teflon-in-glass homogenizer.
(10 full strokes at 1,000 rpm). The homogenate was centrifuged at 1,000×g for 10 min at 4°C, and the supernatant was centrifuged at 48,000×g for 10 min at 4°C to form a mitochondrial-synaptosomal pellet. The pellet was resuspended in 20 volumes of 50 mM Tris-HCl buffer (pH 7.4) and washed three times by centrifugation at 48,000×g for 10 min at 4°C. The final pellet was resuspended to a concentration of about 15 mg protein/ml buffer, and the membrane fraction was stored at −70°C until use. The concentration of protein was determined by the method of Lowry et al. (15) using bovine serum albumin as the standard.

[^3]H NTD binding: Synaptosomal membranes (500 μg protein) were incubated for 60–90 min at 37°C in a total volume of 2 ml of 50 mM Tris-HCl buffer (pH 7.4) with various concentrations of [^3]H NTD (0.1–4 nM) and drugs. After incubation in a dark tube, membrane bound [^3]H NTD was trapped over Whatman glass filters (GF/C), and the precipitates were washed three times with 4 ml aliquots of ice-cold buffer. The filters were placed in scintillation vials with 10 ml of scintillator for at least 6 hr, and the radioactivity was counted by a Packard liquid scintillation counter at an efficiency of 45%.[^3]H NTD binding in the presence of 1 μM nifedipine was defined as nonspecific binding, and this was subtracted from the total binding to obtain specific binding. Nonspecific binding accounted for about 30% of the total binding. The IC50 value (the concentration of drug producing a 50% inhibition of specific[^3]H NTD binding) and the apparent Hill coefficient of the drug were determined by Hill plot analysis (16).

Measurement of vertebral blood flow: Vertebral blood flow was determined in the anesthetized dog as described by Kato et al. (17). Mongrel dogs of either sex (11–18 kg) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). The dogs were ventilated with room air by a respirator (SN-480-4, Shinano). The right vertebral artery was isolated from the surrounding tissues, and the vertebral blood flow was measured continuously with an electromagnetic flow meter (MFV-2100, Nihon Kohden). The drugs were injected into the right femoral vein. The vasodilator activity of the drug was determined as the ED30 value (the dose of drug producing a 30% increase of basal blood flow).

Statistical analysis: Unless specified, the experimental values shown in the text, tables and figures are expressed as the mean±S.E.M. The IC50 and ED30 was calculated using a linear regression analysis program on a personal computer (PC-9801, NEC). Differences were tested for significance by Student’s t-test or the paired t-test, and P＜0.05 was taken as indicating statistical significance.

Drugs: Nifedipine, nimodipine, KB-2796 and other diphenylpiperazines were synthesized in the Synthetic Chemistry Department of Kanebo, Ltd. [5-methyl[^3]H NTD (specific activity, 87 Ci/mmol) was obtained from New England Nuclear. The following drugs were purchased from the companies listed: flunarizine, cinnarizine and nicardipine (Sigma); d-cis-diltiazem (Tanabe); (±)-verapamil (Eisai).

For binding studies, the diphenylpiperazines were dissolved in a 0.1 N HCl solution containing 50% (v/v) ethanol, and 1,4-DHPs were dissolved in 99.5% (v/v) ethanol. d-cis-Diltiazem and (±)-verapamil were dissolved in distilled water. Dilutions of all drugs were made into distilled water. The final ethanol concentration of the incubation buffer was 0.5% (v/v) at maximum. For intravenous administration, the diphenylpiperazines were dissolved in a 20% (v/v) dimethylacetamide solution containing 2% (w/v) tartaric acid at all doses.

Results

Effects of KB-2796 and other calcium antagonists on[^3]H NTD binding: Nimodipine and nicardipine dose-dependently inhibited[^3]H NTD binding to synaptosomal membranes of the guinea pig cerebral cortex, with the IC50 values of 1.7±0.37 and 3.1±0.61 nM, respectively. The Hill coefficients obtained were 1.01±0.05 and 0.93±0.003, respectively. (±)-Verapamil incompletely inhibited[^3]H NTD binding by about 50% at 10 μM. In contrast, d-cis-diltiazem enhanced[^3]H NTD binding, with the enhancement reaching about 200% at 10 μM (Fig. 1).
KB-2796, flunarizine and cinnarizine inhibited \(^{3}\)H NTD binding in apparently the same manner as nimodipine and nicardipine, with the IC50 values being 86±24, 286±54 and 494±100 nM, respectively (Fig. 1 and Table 1). Other diphenylpiperazines (compounds A, B and C) also similarly inhibited \(^{3}\)H NTD binding (data not shown), and the IC50 values are summarized in Table 1. The apparent Hill coefficients of the diphenylpiperazines tested were not significantly different from unity. Among the diphenylpiperazines tested, KB-2796 was the most potent inhibitor.

Effects of KB-2796, flunarizine and cinnarizine on the dissociation rate of \(^{3}\)H NTD: The dissociation rate of \(^{3}\)H NTD was determined by adding 1 \(\mu\)M unlabeled nifedipine to cerebral cortex membranes which had been pre-equilibrated with 0.25 nM \(^{3}\)H NTD. The dissociations of \(^{3}\)H NTD were characterized by a linear relationship between the logarithm of residual binding and the elapsed time (Fig. 3). In the absence of diphenylpiperazines, the dissociation rate constant \((k_{-1})\) was 0.32±0.02 min\(^{-1}\). In the presence of 10 \(\mu\)M KB-2796, 10 \(\mu\)M flunarizine and 10 \(\mu\)M cinnarizine, the \(k_{-1}\) values were significantly increased to 0.90±0.07 min\(^{-1}\) (\(P<0.01\)), 0.95±0.12 min\(^{-1}\) (\(P<0.05\)) and 0.85±0.14 min\(^{-1}\) (\(P<0.05\)), respectively.

Effects of diphenylpiperazines on vertebral blood flow: The diphenylpiperazines tested dose-dependently increased vertebral blood flow in anesthetized dogs (data not shown), and the ED30 values are shown in Table 1.
Table 1. Effects of diphenylpiperazine derivatives on $[^3]$HNTD binding and vertebral blood flow

<table>
<thead>
<tr>
<th>Structure</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>Compound</th>
<th>IC50 ($\mu M$)</th>
<th>nH</th>
<th>n</th>
<th>ED30 ($\mu$mol/kg)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2,3,4-OCH$_3$</td>
<td>4-F</td>
<td>4-F</td>
<td>KB-2796</td>
<td>0.086±0.02</td>
<td>0.96±0.05</td>
<td>4</td>
<td>0.089$^a$</td>
<td>7</td>
</tr>
<tr>
<td>A</td>
<td>3-CH$_3$</td>
<td>3-OH</td>
<td>4-Cl</td>
<td>A</td>
<td>3.07±0.85</td>
<td>1.16±0.22</td>
<td>3</td>
<td>1.81</td>
<td>4</td>
</tr>
<tr>
<td>A</td>
<td>2,3,4-OCH$_3$</td>
<td>4-OCH$_3$</td>
<td>4-OCH$_3$</td>
<td>B</td>
<td>3.84±0.54</td>
<td>0.82±0.24</td>
<td>3</td>
<td>2.30</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>2,3,4-OCH$_3$</td>
<td>4-F</td>
<td>4-F</td>
<td>C</td>
<td>0.094±0.02</td>
<td>0.82±0.14</td>
<td>4</td>
<td>0.081</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>4-F</td>
<td>4-F</td>
<td></td>
<td>Flunarizine</td>
<td>0.286±0.05</td>
<td>0.84±0.09</td>
<td>4</td>
<td>0.314$^a$</td>
<td>7</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td>Cinnarizine</td>
<td>0.494±0.10</td>
<td>0.84±0.13</td>
<td>4</td>
<td>0.380</td>
<td>7</td>
</tr>
</tbody>
</table>

IC50 and Hill coefficient (nH) for $[^3]$HNTD binding and ED30 for vertebral blood flow (VBF) were determined as described in "Materials and Methods". The data for $[^3]$HNTD are the means±S.E.M., and the data for VBF are the means of the indicated number (n) of experiments. $^a$Data from Ohtaka et al. (5).
Table 2. Effects of KB-2796, flunarizine and cinnarizine on the K_d and B_max of [3H]NTD

<table>
<thead>
<tr>
<th></th>
<th>K_d (nM)</th>
<th>B_max (fmole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.51±0.10</td>
<td>144±19</td>
</tr>
<tr>
<td>KB-2796 (0.1 μM)</td>
<td>1.47±0.26^a</td>
<td>159±19</td>
</tr>
<tr>
<td>Flunarizine (0.3 μM)</td>
<td>1.35±0.13^b</td>
<td>155±10</td>
</tr>
<tr>
<td>Cinnarizine (0.5 μM)</td>
<td>1.23±0.08^a</td>
<td>153±3</td>
</tr>
</tbody>
</table>

K_d and B_max were determined by Scatchard analysis. The data are the means±S.E.M. from three experiments, each performed in duplicate. ^aP<0.05, ^bP<0.01, significantly different from the control value.

Fig. 3. Time course of dissociation of [3H]NTD in the absence (control) or presence of 10 μM KB-2796, 10 μM flunarizine or 10 μM cinnarizine. Synaptosomal membranes (500 μg protein) were preincubated with 0.25 nM [3H]NTD for 60 min at 37°C. Dissociation was then initiated by the addition of 1 μM nifedipine. The data are the means±S.E.M. of four experiments, each performed in duplicate. ○ Control, □ KB-2796, △ flunarizine, ▲ cinnarizine.

Among the diphenylpiperazines tested, KB-2796 and compound C showed greater vasodilating effects than the other diphenylpiperazines.

Discussion

In the present study, we characterized the interaction of KB-2796 with the 1,4-DHP binding site in guinea pig cerebral cortex membranes. KB-2796, as well as other diphenylpiperazines, dose-dependently inhibited [3H]NTD binding, with the apparent Hill coefficient being close to unity. Our results obtained with flunarizine and cinnarizine agree well with the previous observation (18). The Scatchard analysis revealed that KB-2796 increased the K_d value of [3H]NTD binding without changing the B_max value. These data indicate that KB-2796 inhibits [3H]NTD binding by decreasing the binding affinity of [3H]NTD. This inhibition profile was similar to that of 1,4-DHP, which is a competitive antagonist (19). However, if one examines the effect of KB-2796 on the dissociation rate of [3H]NTD, seen in Fig. 3, the decrease in affinity of the ligand caused by KB-2796 can be explained by its ability to increase dissociation rate. A competitive antagonist affects the affinity of the ligand by decreasing the apparent association rate without changing the dissociation rate (20). The result of Fig. 3 suggests that KB-2796 regulates the 1,4-DHP binding through a negative allosteric mechanism.

Recent studies (8, 9) have supported the idea that the 1,4-DHP calcium antagonists bind to the voltage-dependent calcium channel. Non-1,4-DHP calcium antagonists, i.e., d-cis-diltiazem, (±)-verapamil, (±)-bepridil and their related compounds, have shown to affect the 1,4-DHP binding by acting at an allosteric binding site (11, 21). In the present study, we also confirmed that (±)-verapamil noncompetitively inhibits [3H]NTD binding, while d-cis-diltiazem stimulates [3H]NTD binding.

Glossmann et al. (8), on the basis of several radioligand binding data, have proposed a three-site model for the binding sites of 1,4-DHPs, verapamil and diltiazem on the voltage-dependent calcium channel. In contrast, the diphenylpiperazines were shown here to affect [3H]NTD binding in a manner different from those of 1,4-DHPs, (±)-verapamil and d-cis-diltiazem. It is assumed that the diphenylpiperazines regulate [3H]NTD binding by acting at a site that is different from those of 1,4-DHPs, verapamil and...
diltiazem on the voltage-dependent calcium channel.

Among the diphenylpiperazines tested, KB-2796 was the most potent inhibitor of $[^{3}H]$NTD binding. Moreover, KB-2796 also possessed a greater vertebral vasodilating effect than flunarizine and cinnarizine in anesthetized dogs. Recently, Dooley et al. (22) reported that the 1,4-DHP binding sites in rat neocortical synaptosomes and microvessels are similar. Thus, the potent cerebral vasodilating activity of KB-2796 might reflect its potent inhibiting activity for $[^{3}H]$NTD binding.

In conclusion, the present study indicates that KB-2796 inhibits the 1,4-DHP binding by a negative heterotropic allosteric mechanism. This finding suggests that KB-2796 acts at a site, which is distinct from the 1,4-DHP binding site, on the voltage-dependent calcium channel. In order to analyze the detailed properties of the binding site for KB-2796, however, further studies are needed.

**References**

KB-2796 and [³H]Nitrendipine Binding

550 (1985)

